Reference: *Biol. Bull.*, **147**: 136–145. (August, 1974)

HARVEST OF PLANKTONIC MARINE ALGAE BY CENTRIFUGATION INTO GRADIENTS OF SILICA IN THE CF-6 CONTINUOUS-FLOW ZONAL ROTOR[1]

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The harvest of the more common algae from laboratory cultures rarely presents serious problems. However, the collection (or concentration) of living algal cells in good physiological condition from dilute cultures or from natural plankton populations is much more difficult. The two major devices commonly used are filtration and centrifugation. Filtration is carried out commonly on membranes of modified cellulose (Clarke and Sigler, 1963), with the aid of a suction pump. The greatest advantage of this method as a concentrating device is that it is able to collect microalgae or cells of very low density. However, concentration by filtration is limited to small volumes and leads to the eventual clogging of the filter by the packed cells when vacuum is applied.

Several methods have been devised which avoid these problems. One involves the use of a reverse-flow vacuum (Dodson and Thomas, 1964) in which the pressure operates from above, making the process more gentle and avoiding the packing of cells. This method itself has been modified to allow a relatively large volume of water to be concentrated in a short period of time (20 liters to 300 ml in 3 hours) (Holm-Hansen, Packard and Pomeroy, 1970). A second process uses a direct vacuum but involves a stirring blade in the flask above the filter which prevents the particles from settling at all during the concentration process (Morris and Yentch, 1972).

Continuous-flow centrifugation with the classical Foerst rotor or the Szent-Gyorgyi-Blum modification of the Sorvall rotor is another widely used method. This method is reasonably efficient, but sensitive algal cells may be damaged by pelleting against the rotor wall and the method is essentially unselective; all particles with a sedimentation rate above some limiting value will be collected.

The variant on zonal centrifugation known as continuous sample-flow with isopycnic banding (*cf.* Cline and Ryel, 1971) offers a number of theoretical advantages in the concentration (and simultaneous purification) of particles: large capacity, more efficient recovery at substantially lower speeds than are required for conventional continuous-flow centrifugation, and avoidance of pelleting. Plankton, including algae, have been collected in sucrose gradients in the B-XVI and K-II zonal rotors (Lammers, 1971), but we do not know either the efficiency of recovery or the integrity of the recovered algae.

[1] Supported in part by a contract from the MARMAP Program of the National Marine Fisheries Service and an NSF Grant (GA 33288) to R. R. L. Guillard; paper of the Journal Series, New Jersey Agricultural Experiment Station, Cook College, Rutgers University, New Brunswick, New Jersey 08903; contribution no. 3252 from the Woods Hole Oceanographic Institution.

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TABLE I

Algae investigated

Species	Clone	Class	Approximate dimensions	Origin
Dunaliella tertiolecta	Dun	Chlorophyceae	6.5 × 10 μ	Unknown; possibly derived from Plymouth #83
Pyramimonas sp.	Pyr-I	Prasinophyceae	8 × 15 μ	San Francisco, California
Thalassiosira fluviatilis	Actin	Bacillariophyceae	20 μ (isodiametric)	Long Island Sound (Gardiner's Island)
Synechococcus bacillaris	Syn	Cyanophyceae	0.75 × 1.5 μ	Milford, Connecticut

In any event it should be possible to harvest algae with centrifuge systems which are much slower and less costly than the zonal centrifuges designed for the purification of viruses. We have, therefore, tested an alternative means of harvesting algal plankton in the CF-6 rotor (Casciato, 1973), a continuous-flow zonal rotor.

MATERIALS AND METHODS

Organisms and conditions of growth

The algal strains used in this work are listed in Table I, together with clonal designation, algal class, origin, and approximate size of each. Algae will be referred to by clonal designation in this paper. All clones are maintained by R. R. L. Guillard at the Woods Hole Oceanographic Institution. These particular species are taxonomically varied and cover a range of sizes, though all are in the smallest size class of the phytoplankton (the nannoplankton). Two clones (Dun and Pyr-I) are naked flagellates; the blue-green algal clone Syn has a multilayered wall with protein, carbohydrate, and mucopeptide components; the diatom Actin has a wall composed of a silica shell in addition to an organic matrix. We expected the structural variability to influence the density during centrifugation. The chlorophyte Dun was particularly useful because of its motility and general hardiness; a visual indication of its viability under gradient conditions and manipulation could be obtained by observing motility.

All algae were grown in an enriched artificial seawater. A seawater base was made by using the major constituents ($MgSO_4$, $MgCl_2$, $CaCl_2$, KCl, $NaHCO_3$, and NaCl) of ASP-M (McLachlan, 1964) plus 1 ml/l of an 0.6% (w/v) solution of H_3BO_3. This seawater base was then supplied with the nutrients of enrichment "f" (Guillard and Ryther, 1962) with all nutrients at half the concentrations specified.

Cultures were grown at 20–24°C under continuous illumination of 3500–6000 lux from cool-white (Sylvania Co.) fluorescent lamps. Cultures were grown in Erlenmeyer flasks or carboys with aeration.

Preparation of gradients

The silica gradients were prepared as follows: Ludox AM (E. I. du Pont de Nemours and Co., Wilmington, Delaware), a preparation of colloidal silica with

partial substitution of Si by Al, was allowed to dialyze against distilled water for at least two weeks; water was changed daily. The Ludox was then collected and used for several runs. The reason for this dialysis is that, contrary to the evidence of others with animal cells (Mateyko and Kopac, 1963; Pertoft, 1969), Ludox AM was found to be highly toxic to algal cells, marine algae as well as *Euglena gracilis*. We suspect that the toxicity is due to the presence of a biocide in the commercial product. Two weeks of dialysis does not completely remove the toxic quality

The gradients used were: 40% to 70% v/v dialyzed Ludox for Dun, Pyr-I, Syn and 20% to 50% v/v dialyzed Ludox for Actin (see graphs in Results). Gradients of zero to 3% w/v NaCl ran counter to the Ludox to minimize gelling. The appropriate gradients were established by trial runs with step gradients in swinging buckets. The banding densities were monitored by the use of calibrated density beads (Clark Wilcox and Assoc., Los Altos, California). Gradients were also prepared from mixtures of Ficoll in seawater. Ficoll is a trade name for polysucrose, available from Pharmacia Fine Chemicals, New Market, New Jersey.

Harvest and centrifugation

Cells were harvested in the 620-ml CF-6 zonal rotor (IEC/Damon, Needham Heights, Massachusetts), in the PR-6 (IEC) centrifuge. The gradient consisted of 235 ml of starting solution, 220 ml of limiting solution and 115 ml of underlay. The starting and limiting solutions were mixed in the IEC gradient former and pump (#3651) to give a gradient which was linear with volume, and were pumped into the rotor at 40 ml/min. The rotor speed during loading was 1500 rpm. The sample was loaded at 100 or 200 ml/min by either the Harvard (#600–00) or the Cole-Parmer Masterflex (#WZ-1R031) peristaltic pump. The sample was then run for 15 min at 4000 rpm to band the particles, slowed to 1000 rpm, and the gradient displaced by pumping at 35 ml/min. The gradient was collected in 30-ml fractions in chilled graduated test tubes.

In order to study the efficiency of collection, samples of the algal suspensions were fixed with 1% glutaraldehyde and the cell concentrations determined subsequently in a Coulter Counter by Dr. J.-J. Morgenthaler. Background counts were taken for comparison with counts from supernatants and original culture samples (see Table II).

Analyses

Growth. Relative cell concentration was measured by fluorometry of the chlorophyll (Knight, 1968). A 436 nm interference primary filter and a #66 Klett secondary filter were used in a Turner Model III fluorometer. Fluorescence was found to be proportional to cell number. Daily samples of about 4 ml were taken aseptically from the cultures in 250-ml culture flasks. Where dilution was necessary, it was done with artificial seawater. The blank was also artificial seawater. Growth rates and progress curves were obtained by plotting relative fluorescence against time on a semilog scale (Fig. 3 a–d).

Chlorophyll. The concentrations of cell material within the peak zones of the gradient were established by measuring the amounts of chlorophyll spectrophoto-

metrically. This method was also used to compare the chlorophyll content of the original culture suspension with that of the harvested cells. One milliliter from a 30-ml gradient fraction or 100 ml of the culture suspension concentrated to 1 ml by centrifugation were placed in 4 ml of 100% acetone and allowed to remain in the cold and dark for 1/2 hour. They were then centrifuged at 2000 rpm for 10 min and the absorbance of the supernatant read at 652 nm (Arnon, 1949; Bruinsma, 1961).

Polarography. Oxygen polarography was used to determine the rates of photosynthesis and respiration of harvested and unharvested cells. Harvested cells from the tube corresponding to the peak concentration of cells were diluted 1 : 10 or 1 : 5 in artificial seawater or 100 ml of unharvested cells were centrifuged at 2000 rpm for 10 min and resuspended in 3 ml of medium. Oxygen exchange by the algal suspensions in the dark (respiration) and in the light (net photosynthesis) were measured with the Model 53 Biological Oxygen Monitor (Yellow Springs Instrument Company, Yellow Springs, Ohio).

Results

Efficiency of collection

The efficiency of collection of algae by the rotor was measured by comparing the concentration of algae in the original suspension with that in the effluent from the rotor. The four organisms studied cover a range of sizes that include most planktonic forms. The clean-out at any given speed is a function of the size of the alga (Table II). On this basis we have projected the flow rates required to yield $\geq$ 90% clean-out. For the larger algae at 1500 rpm and 200 ml/min, clean-out was essentially quantitative, and flow rates can probably be increased severalfold without significant decrease in clean-out. For *Synechococcus*, which is of bacterial size, the clean-out was no better than 78% at maximum rotor speed and at the lower flow rate. *Synechococcus* may be at the lower limit of particle

Table II

Clean-out of algal suspensions in the CF-6. Fractional "clean-out" is a measure of the efficiency of retention of particles in a continuous-flow rotor and defined as

$$\left[\frac{\textit{original concentration} - \textit{effluent concentration}}{\textit{original concentration}}\right]$$

The coefficient of variation of clean-out varied from 0.2 to 8%

Organism		Flow-rate ml/min	Cell number/ml		Fractional clean-out
			Initial	Effluent	
Dun	6.5 × 10 μ	200	137,767	968	0.99
Actin	10–20 μ	200	50,160	111	0.99
Pyr-I	8 × 15 μ	200	71,585	269	0.99
Syn*	0.75 × 1.5 μ	100	8,430	1818	0.78
		150	8,430	1790	0.78
		200	8,430	3115	0.63

* Determined at rotor speed of 6000 rpm. All others at 1500 rpm.

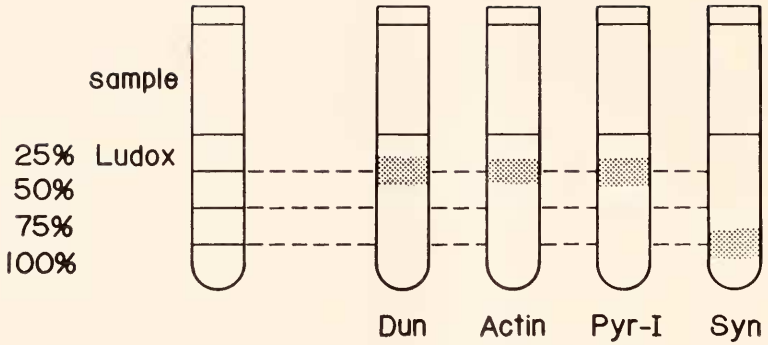

FIGURE 1. Diagram of isopycnic banding of different algae in step gradients of Ludox.

size that can be separated under the present conditions. Very few algal species are as small as *S. bacillaris*.

Equilibrium densities

The banding densities were initially estimated in step gradients of silica in swinging buckets. After centrifugation at 5000 rpm for 5 min, the banding patterns sketched in Figure 1 were obtained. These measurements showed that Dun, Actin and Pyr-I banded at approximately the same densities, while Syn banded at a much higher density.

More precise distributions were determined from direct measurements in the continuous gradients recovered from the CF-6. The volumes in which the cells were recovered were in the order of 100 ml (Fig. 2 a–d), but substantial heterogeneity in density was sometimes observed. Dun banded between $\rho = 1.07$–1.12, but principally near 1.08. Actin banded between 1.09–1.12.

Integrity

Any method of harvest is of limited usefulness if the cells cannot be recovered physiologically intact and viable.

We tested physiological state by polarographic measurements of respiration and photosynthesis, before and after harvest, for some of the algae. A summary of the data is presented in Table III. In the case of Dun, there was no loss of photosynthetic or respiratory activity after banding in Ludox gradients. For the other species, both the rates of photosynthesis and respiration were decreased to 1/3 to 1/5 of the original values after the cells were harvested from the Ludox gradients. To determine whether Ludox itself affected photosynthesis or respiration, separate experiments were run whereby polarography was measured on cells in the absence or presence of the silica sol ($\simeq 66\%$ Ludox) using the organisms Dun and Syn. In both cases there was an enhancement (doubling) of respiration in the presence of Ludox. However, photosynthesis was again lowered to about 1/4 in the case of Syn and essentially unchanged in the case of Dun, in the presence of Ludox. There have been reports that Ludox catalyzes certain biological

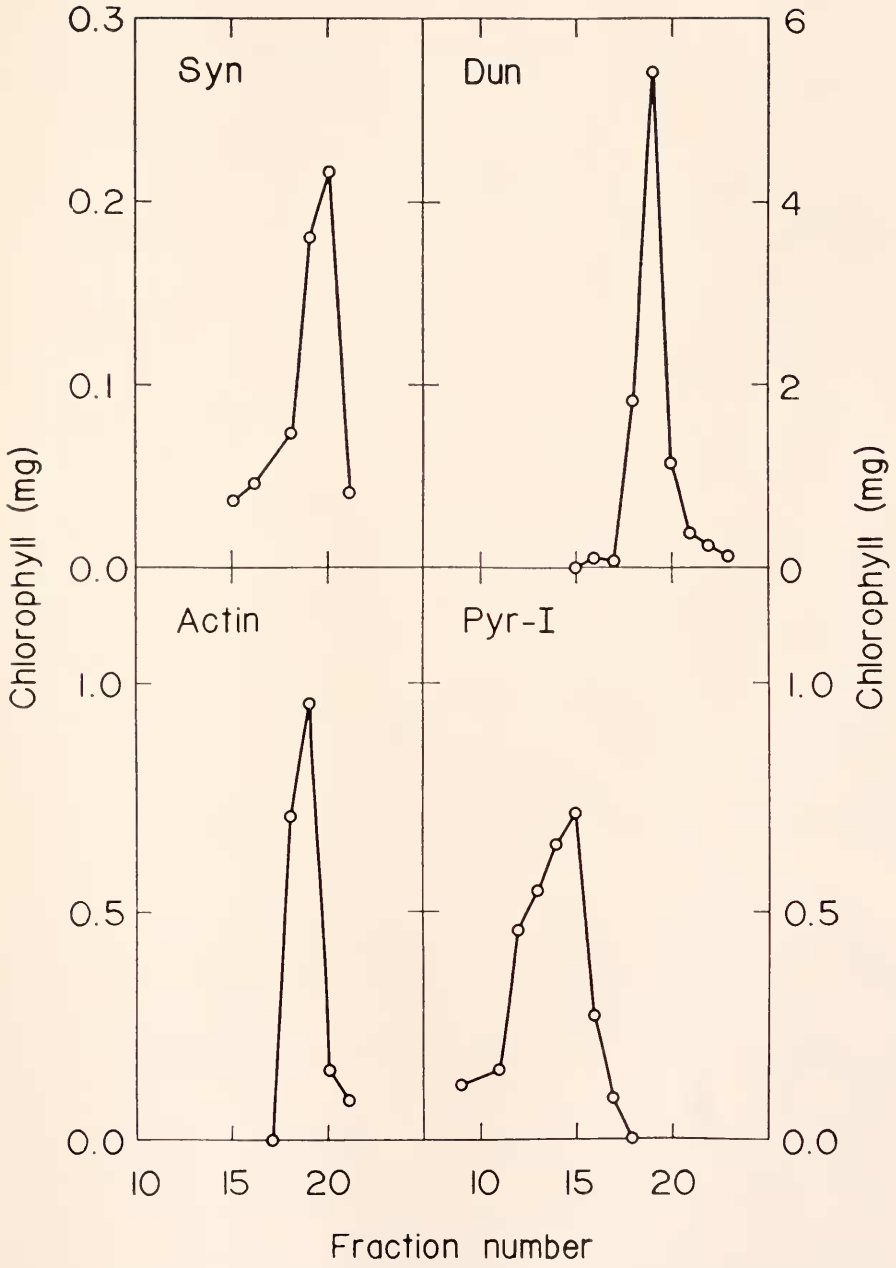

FIGURE 2. Recovery of algae after continuous sample flow with isopycnic banding in gradients of Ludox. Details of the method are described in the text. Cell concentration was estimated by monitoring the absorbance of the fractions at 652 nm; *Synechococcus bacillaris* (Syn); *Dunaliella tertiolecta* (Dun.); *Thalassiosira fluviatilis* (Actin); *Pyramimonas* sp. (Pyr-I).

TABLE III

Respiration and photosynthesis of algae before and after centrifugation through Ludox gradients

Sample	Rates of O_2 exchange nmoles O_2 $hr^{-1} \cdot \mu g$ chlorophyll^{-1}	
	Dark	Light
Pyr-I (83/52)		
Original	− 61.5	+ 21
Ludox-harvested	− 23.2	+ 7.8
Syn (83/54)		
Original	−229	+285
Ludox-harvested	− 89	+ 52
Dun (83/57–SB)		
Original	− 12.6	+136
Ludox-harvested	− 12.6	+210

oxidations (Slawson, Adamson and Mead, 1973). This may account for the observed increase in O_2 uptake by the cells in the presence of concentrated amounts of Ludox.

Viability was measured by inoculating fresh media with approximately equal numbers of cells before and after harvest. We measured growth from the rate of increase of chlorophyll; although this method will not necessarily provide a measure of absolute growth rates, the existence of an abnormal lag phase will indicate if there are significant numbers of cells which are nonliving or whose growth rates have been rendered abnormal. Growth appeared to proceed normally except in the case of Syn, which showed a significant lag period (Fig. 3 a–d).

DISCUSSION

We have shown that several kinds of planktonic algae can be harvested from laboratory cultures by continuous-flow centrifugation into gradients of silica. The recovered cells are not seriously damaged in the process, as shown by their essentially normal rates of growth in fresh medium. The rates of sample flow are such that 50 liters of culture of the smallest algal cells could be harvested in a four-hour interval. The absolute quantities are limited only by the capacity of the gradient, which is of the order of tens of grams of cells.

Density gradient centrifugation is performed most often using sucrose as the gradient material. However, sucrose has the distinct disadvantage that the high osmotic potential of sucrose solutions will plasmolyze whole cells. Plasmolysis, in addition to the danger of working irreversible changes on the cells, has the effect of increasing the cell density. In fact, we found that none of our organisms could be floated in any concentration of sucrose-seawater up to 65% w/w nor in sorbitol-seawater up to 60% w/w.

Ficoll-seawater did provide a medium in which the cells could float and remain approximately normal in size and shape. We found that algae required up to 25%

w/w Ficoll in seawater, a solution that is extremely viscous and difficult to pump in and out of the rotors; in addition it is expensive.

Ludox, a silica sol, is non-osmotic like Ficoll, but poses neither the problem of viscosity nor expense. However, it has some peculiarities of its own: It has a tendency to gel in the presence of salts. We found that low concentrations of Ludox were stable in 3% w/v NaCl, but higher concentrations were not. We were obliged therefore to employ Ludox gradients in which the dense end of the gradient is deficient in salt with respect to ordinary seawater. For stenohaline organisms

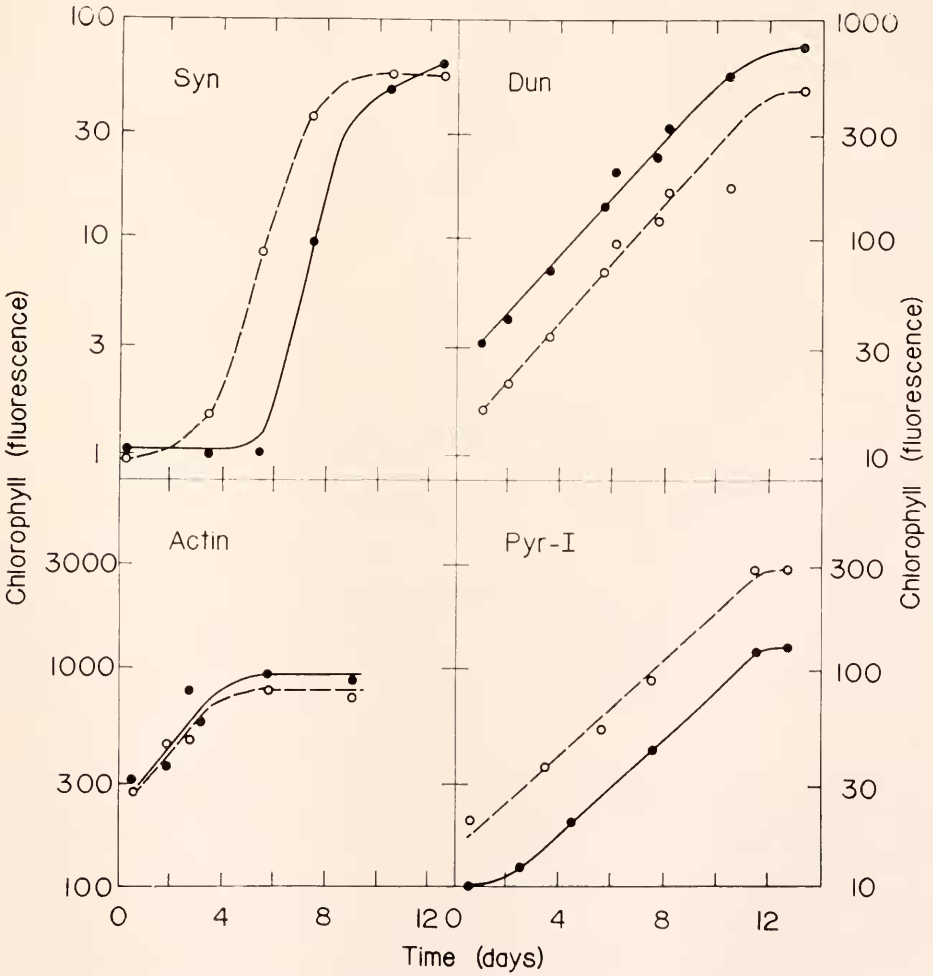

FIGURE 3. Comparison of growth rates of algae before and after harvest by centrifugation in Ludox gradients. Cell densities were estimated by the relative concentration of chlorophyll as determined by fluorescence. Similar numbers of cells were inoculated into fresh media at zero time. The absence of an abnormal lag period was taken as an indication of viability; *Synechococcus bacillaris* (Syn); *Dunaliella tertiolecta* (Dun); *Thalassiosira fluviatilis* (Actin); *Pyramimonas* sp. (Pyr-I).

the deficit in osmotic potential can be compensated with sorbitol or other compatible osmotica. We have also found some evidence of toxicity of Ludox.

The movements of flagellates, such as *Dunaliella,* ceased abruptly when exposed to even a low concentration of ordinary Ludox. We then found that this toxicity is removed by dialysis, but prolonged dialysis may substantially dilute the preparation. Silica sols free of added biocides have recently become available from Nalco Chemical Company, Chicago, Illinois. In some cases biological activity may be preserved by the addition of small amounts of polymers such as polyethylene glycol (Pertoft, 1969; Morgenthaler *et al.*, 1974).

The lowered rates of respiration and photosynthesis obtained after the organisms were harvested from Ludox gradients may, as in the case of Pyr-I, reflect short-term physiological effects, since growth appeared to proceed normally or after a brief lag period. Of the organisms tried here, Dun seemed to be the least affected by centrifugation in gradients of Ludox, whereas Syn manifested physiological damage, as shown by the increased lag period as well as decreased rates in respiration and photosynthesis. Since Dun occurs naturally in brackish waters, its insensitivity to Ludox gradients may be related to its tolerance of low concentrations of salt.

Summary

1. Cells of four different phytoplankton species were harvested from cultures using gradients of colloidal silica (Ludox) in the CF-6, a continuous-flow zonal rotor.

2. The efficiency of harvesting ("fractional clean-out") was > 0.99 for three of the algae and > 0.78 for the smallest species (*Synechococcus bacillaris*).

3. Growth rates of the algae subcultured after harvesting in Ludox were similar to those unharvested controls, but *Synechococcus* showed a lag period of about four days after harvesting.

4. The rates of photosynthesis and respiration of cells harvested in Ludox gradients were, in most cases, about $\frac{1}{3}$ to $\frac{1}{5}$ of the pre-harvest rates. *Dunaliella tertiolecta* showed no loss of photosynthetic or respiratory activity.

5. This method has application to the collection of algae from dilute cultures and from natural waters when cells are needed in good physiological condition.

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